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TRAIL-MARKING AND ALARM PHEROMONES
OF SOME ANTS OF THE GENUS ATTA

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This compound

The volatile trail-marking substance of Atta texana has been identified as methyl-4-methylpyrrole-2-carboxylate.

The isolation and identification of the non-volatile trail pheromone of Atta texana has led to inconclusive results due mainly to erratic bioassay results.

Current laboratory research is being focused on the isolation and identification of the volatile trail pheromone(s) of Atta cephalotes. A synthesis of both enantiomers of 4-methyl-3-heptanone is in progress. Their biological activity will be tested on Atta texana and Atta cephalotes following successful completion of the synthesis. (EPA)

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I Past and Present Research in Ant Pheromones

A. Introduction:

Fungus growing ants are found only in the New World, but they range from New Jersey to California and South to Argentina. Collectively known as the Tribe Attini, they number about 250 species, of which 13 are in the United States. The feature that chiefly distinguishes them from all other ants is the underground gardens in which they cultivate a fungus that provides their chief food (Weber, 1966). The leaves and other materials they forage form a substrate for the fungus. There are two types of pheromones that conspicuously modulate their behavior; the alarm pheromones and the trail-marking pheromones; it is with these that our research has been concerned.

B. 4-methyl-3-heptanone, an Alarm Pheromone of the Ant Sub-family Myrmicinae.

4-methyl-3-heptanone has been identified as an alarm pheromone for many species of ants of the sub-family Myrmicinae. Within this sub-family, species of the three genera, Atta, Trachymyrmex, and Pogonomyrmex have been found to possess the compound. McGurk et. al., (1966) was the first to identify the compound from the mandibular glands of three species of Pogonomyrmex ants. Moser et. al., (1967) identified the ketone along with 2-heptanone as the major volatile constituents of the leaf-cutting ant Atta texana. Blum et. al., (1967) undertook an investigation of the constituents of the mandibular glands of six species of Atta and found all of them to contain 4-methyl-3-heptanone in varying concentrations. In a more

recent publication, in an attempt to correlate the phylogeny of four genera in the sub-family Myrmicinae, Crewe et. al., (1971) identified 4-methyl-3-heptanone as a major component of the mandibular gland extract of Trachymyrmex seminole.

C. Source and Behavior of Ants to Alarm Pheromones.

The source of the volatile alarm substances of Atta are the mandibular glands located in the head of the ant. When an ant is disturbed, usually by an outside intruder, the volatile alarm substances are discharged from these glands as a vapor. Typical alarm behavior includes raising of the head and antennae, raising upon their back legs, opening up of their mandibles and running around at random challenging other ants.

D. Alarm Activity and Chemical Structure.

Two studies on different ant systems have been conducted in recent years in an attempt to relate the alarm activity of naturally occurring compounds to their chemical structures. Blum et. al., (1965) tested the alarm activity of 49 ketones on Iridomyrmex pruinosus using 2-heptanone (the known alarm pheromone of I. pruinosus) as the standard in the study. Blum concluded from these trials that alarm activity is a function of the length and degree of branching of the carbon skeleton and position of the carbonyl group in the molecule. Moser et. al., (1967) conducted similar tests with Atta texana using 4-methyl-3-heptanone and 2-heptanone (the alarm pheromones of Atta texana) as standards which arrived at similar conclusions.

Although past experimental evidence has established the significance of chemical structure and alarm activity, no investigations to date have studied the effect that the absolute configuration of a molecule has on the magnitude of the alarm response from a given specie of ant. Since 4-methyl-3-heptanone has a center of asymmetry, it lends itself very nicely to such a study.

4-methyl-3-hepanone exists as a pair of enantiomers. If we consider the receptor sites on the antennae of the ant which sense the molecules of alarm pheromone and assume that these receptor sites are asymmetric or "enzyme like", it could be postulated that both forms of 4-methyl-3-heptanone do not give the same degree of alarm response when bioassayed at the same concentration levels. The interaction of the asymmetric receptor sites with each enantiomer of 4-methyl-3-heptanone produces interactions which are diastereomeric (physically different) and therefore should produce different levels of alarm response for each enantiomer. Depending on the degree of selectivity of the receptor sites, (if the receptor sites have the same degree of selectivity as enzymes) it is possible that one enantiomer is highly active and the other totally inactive.

E. Source and Potency of Odor-Trail Pheromones.

The basic orodor-trail substance is presumably similar in all Attini. In the laboratory, specialized species such as those of the genus Atta readily follow trails marked by substances from the less complex genera of the tribe. In the field, trails of various species cross, but there is no evidence of trail-sharing. Indeed, when ants

of one species are placed on the trail of another, they wander about until they encounter their own trail. Possibly each species produces another chemical, in addition to the basic odor-trail substance, that attracts only members of its own species. Non-attines do not follow the trail substances of the attines (Blum et. al., 1964), but Blum and Portocorrero (1966) showed that two genera, at least, follow artificial trails of a non-attine, Daceton, which does not lay trails.

The main odor-trail pheromone is secreted by the gland that produces poison in other ant species. That is, the mechanism once probably used by the Attini for defense or attack has evolved into a device whose sole function is communication. The development parallels the biology of the insects, which are vegetarians. Most other ants of the sub-family Myrmicinae (to which the Attini belong) have retained functional stings, but these species are either scavengers or predators; the imported fire ant is a notable example (Moser, 1963).

The structure of the attine poison apparatus is almost identical in four representative genera examined, which is evidence of a close relationship among species of the group. The odor substance is formed in the true poison gland and stored in the poison sac, a bladder-like structure with a crinkly surface. A tube connects this sac to the stinger. A large Dufour's gland is also connected to the stinger, but its function is unknown. This gland supplies the basic odor substance of fire ants (Wilson, 1959) and perhaps furnishes a secondary substance that distinguishes the trails of attines (Blum et. al., 1964).

The point is speculative. The secondary substance, if it exists, may be an anal emission.

In Atta texana, poison sacs first appear in pupae. At that state the sacs are empty and, when macerated, do not attract workers on artificial trails. Sacs in young adult workers are partially full and the contents have limited potency; the sacs are full after the insect achieves its full red color. Queens, both virgin and fertile, have a complete poison apparatus, but the sacs are always empty. Males have no poison apparatus. Both males and queens avidly follow odor trails in the laboratory (Moser, 1963).

The poison sac substance that attines lay on trails is a clear liquid, soluble in methylene chloride, carbon tetrachloride, or chloroform. It forms a milky-white suspension in acetone, alcohol, or water. Only a fraction of the contents, however, may be the actual odor-trail pheromone.

II Summary:

A. Alarm Pheromones of Atta Texana

The isolation and identification of the alarm pheromones, 4-methyl-3-heptanone and 2-heptanone from the mandibular glands of Atta texana was reported by Moser et. al., in 1967.

Approximately 3.7 kilograms of Atta texana workers were collected in Louisiana near the Alexandria Forestry Center under the supervision of Dr. John Moser. Earlier studies were carried out

with dissected poison sacs to delineate the source of the trail marking substance and to devise a suitable bioassay procedure. To obtain enough material for isolation and identification, whole ants were extracted in a Waring blender with methylene chloride. The extract, after removal of the solvent, was distilled under high vacuum in a short path distillation apparatus. The residue from the distillation was active, and, after volatile alarm substances were removed by liquid chromatography, the distillate was also active. The principal alarm substance (from the mandibular glands) was identified as 4-methyl-3-heptanone, which was detectable by a laboratory colony at a vapor phase concentration of about 33×10^6 molecules/cm³. Another much less active component, 2-heptanone, was also identified (Moser and Silverstein, 1967, Moser et. al., 1968, and Moser, 1970).

B. The Volatile Trail Pheromone of *Atta texana*.

Use of this same basic technique resulted in the isolation, identification, and synthesis of the first ant trail pheromone to be reported (Tumlinson et. al., 1971). The major volatile component of the trail marking substance of *Atta texana* is methyl 4-methylpyrrole-2-carboxylate.

The first synthesis sequence yielded 18 mg. of the pheromone; this was sent to Dr. Moser, who reported that minor workers from the laboratory colony responded at the level of 0.08×10^{-12} grams/cm. of trail (3.48×10^8 molecules/cm.). Thus only 0.33 mg would be

required to lay a detectable ant trail around the world.

Medium and large workers were then tested on natural trails leading from nests in the field in Grand Parish, Louisiana. They readily followed a 2.7×10^{-12} gram/cm. trail on a 15 cm. strip of cardboard placed across an "erased" portion of the trail. They detected and followed another trail made by dribbling 4.0×10^{-12} gram/ul. on the sand at a 45° angle to the field trail. Response was strong at 40.0×10^{-12} gram./ul. A description of the isolation, identification, and synthesis and laboratory and field studies is published in a paper (Tumlinson et. al., 1971).

C. The Non-Volatile Trail Pheromone of *Atta texana*.

Work on the non-volatile trail pheromone of *Atta texana* has led to inconclusive results. This study has been plagued by erratic results. An isolation scheme involving an extensive use of liquid solid chromatography yielded two components from the active fraction, which when bioassayed at various concentration levels led to indefinite conclusions due to the weakness of the biological activity and non-reproducibility of the activity.

D. Optical Activity and the Biological Activity of Alarm Pheromones.

Recently, successful isolation and identification of (+)-4-methyl-3-heptanone from both *Atta texana* and *Atta cephalotes* has been achieved. Current laboratory work is being focused on synthesis of both enantiomers of 4-methyl-3-heptanone in order that their biological activity can be compared.

E. The Volatile Trail Pheromone(s) of *Atta cephalotes*.

Laboratory studies are presently being conducted on the isolation and identification of the volatile trail pheromone(s) of *Atta cephalotes* using the methodology that was applied to *Atta texana*. Because of the difficulties of collecting large quantities of starting material in the tropical rain forests of Costa Rica, we were only able to obtain, over a period of one year, approximately 5 lbs. (2.3 kg.) of *Atta cephalotes*. Our original goal was 20 lbs. of ants. Because of the restriction on the quantity of starting material, it was deemed necessary to employ a certain work-up procedure that was not used in the isolation and identification of the volatile trail pheromone of *Atta texana*. In order to simplify the work-up of the crude volatile trail pheromone active extract, the heads of the ants were removed manually, thus reducing the amount of material that had to be extracted from 2.5 kilograms to approximately 1.1 kilograms. This laborious but simple dissection removed many volatile constituents that would have necessitated removal at a later time by liquid chromatography and gas chromatography. This dissection also removed the interfering alarm pheromones from the crude extract which simplified the bioassay for trail pheromone activity.

From this point, the isolation procedure was similar to the procedure employed for *Atta texana*. The crude material was extracted with methylene chloride; the solvent was removed by fractional distillation at atmospheric pressure and the entire volatile extract

was chromatographed through the first G.L.C. column. Preliminary bioassay studies established the trail pheromone activity in one nine minute fraction from the first G.L.C. column. (5% SE-30 on Chromosorb W 60/80 mesh, 2 1/2 ft. x 1/4 inch). At this point, the isolation work had to be halted because our collaborators in Costa Rica, who were doing the bioassay work, had to return to the United States. They are returning to Costa Rica in March 1973 at which time the isolation of the volatile trail pheromone(s) will be completed and its structure determined.

III Experimental:

A. The Alarm Pheromones of *Atta texana*.

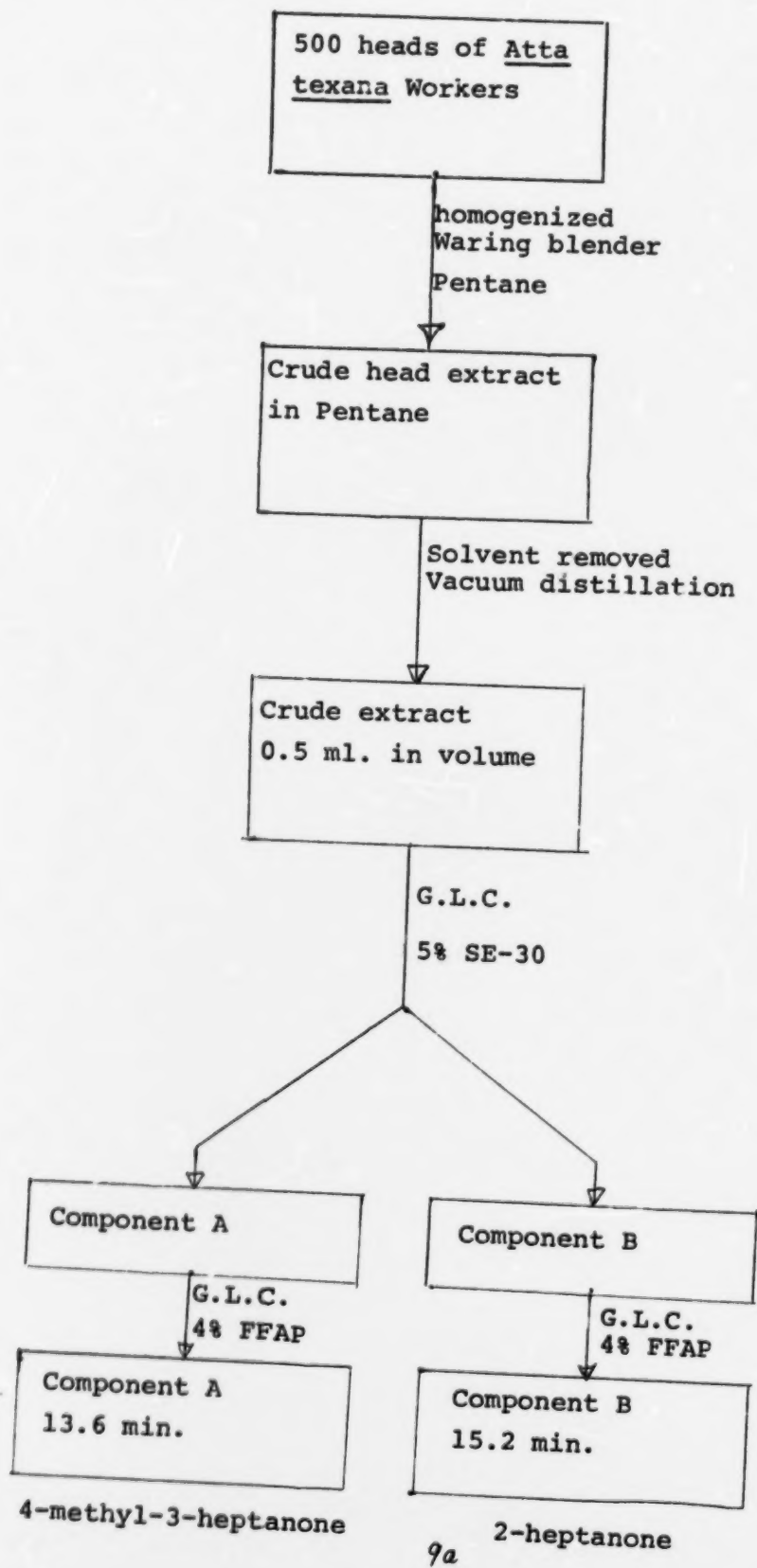
Pheromone research on *Atta texana* began with the isolation of 4-methyl-3-heptanone and 2-heptanone from the mandibular glands in the head of the ant.

1. Isolation and Identification of the Alarm Pheromones.

The heads from 500 worker ants of *Atta texana* were homogenized in a Waring blender using pentane as a solvent. The extract was collected and solvent removed under reduced pressure to a volume of 0.5 ml. The extract was injected on a G.L.C. column (5% SE-30 on chromosorb G 60/80 mesh, 5 ft. x 1/8 in., Helium flow rate 30 cm³/min.) temperature programmed from 40°C to 150°C at a rate of 2.5°C/min. Two major components were collected and rechromatographed on 4% FFAP on Chromosorb G 60/80 mesh, 10 ft. x 1/8 in, He flow rate 10 cm³/min., 60° isothermal. Two major fractions at 13.6 and 15.2 min. were collected and found to

Figure 1

Isolation of Volatile Alarm Pheromones of Atta texana



correspond to 4-methyl-3-heptanone and 2-heptanone when compared with authentic samples of the two ketones. Concentration studies showed 4-methyl-3-heptanone to be present to the extent of 0.59 µg/head and 2-heptanone to be present to the extent of 0.14 µg/head.

Other compounds, 4-methyl-3-heptanol, 2-heptanol, citronellal, geranial and neral which have been previously reported in Hymenoptera were not present.

2. Biological Activity of the Alarm Pheromones.

The threshold concentrations of both ketones were determined in the following manner: The air in two 250 ml flasks was saturated with the vapors of each ketone and one milliliter of head space was drawn up in a ten milliliter syringe and injected through a serum cap fitting into the colony chamber. The initial head space gas was serially diluted with air in the syringe until threshold concentration levels were achieved. The reaction of the ants were observed and the following bioassay criteria were established:

Detection: Worker ants raised their heads and antennae.

Attraction: Workers climbed the walls of the chamber to a height of three cm. or more and moved towards the side of the chamber.

Alarm: Workers run about at random, mandibles open challenging other ants.

Bioassay results showed 4-methyl-3-heptanone to be detected at a syringe serial dilution of 1:1,000,000 and alarm resulted at a serial dilution of 1:100,000. 2-heptanone was about

1000 times less active as 4-methyl-3-heptanone. Tests of other carbonyl compounds showed none of them to be as active as 4-methyl-3-heptanone.

B. The Volatile Trail Pheromone of *Atta texana*.

1. Detection of the Volatile Trail Pheromone

Laboratory colonies of *Atta texana* were observed and found to lay a trail from the colony to the source of food. The ants were observed to touch their abdomens to the ground at intervals depositing the trail substance.

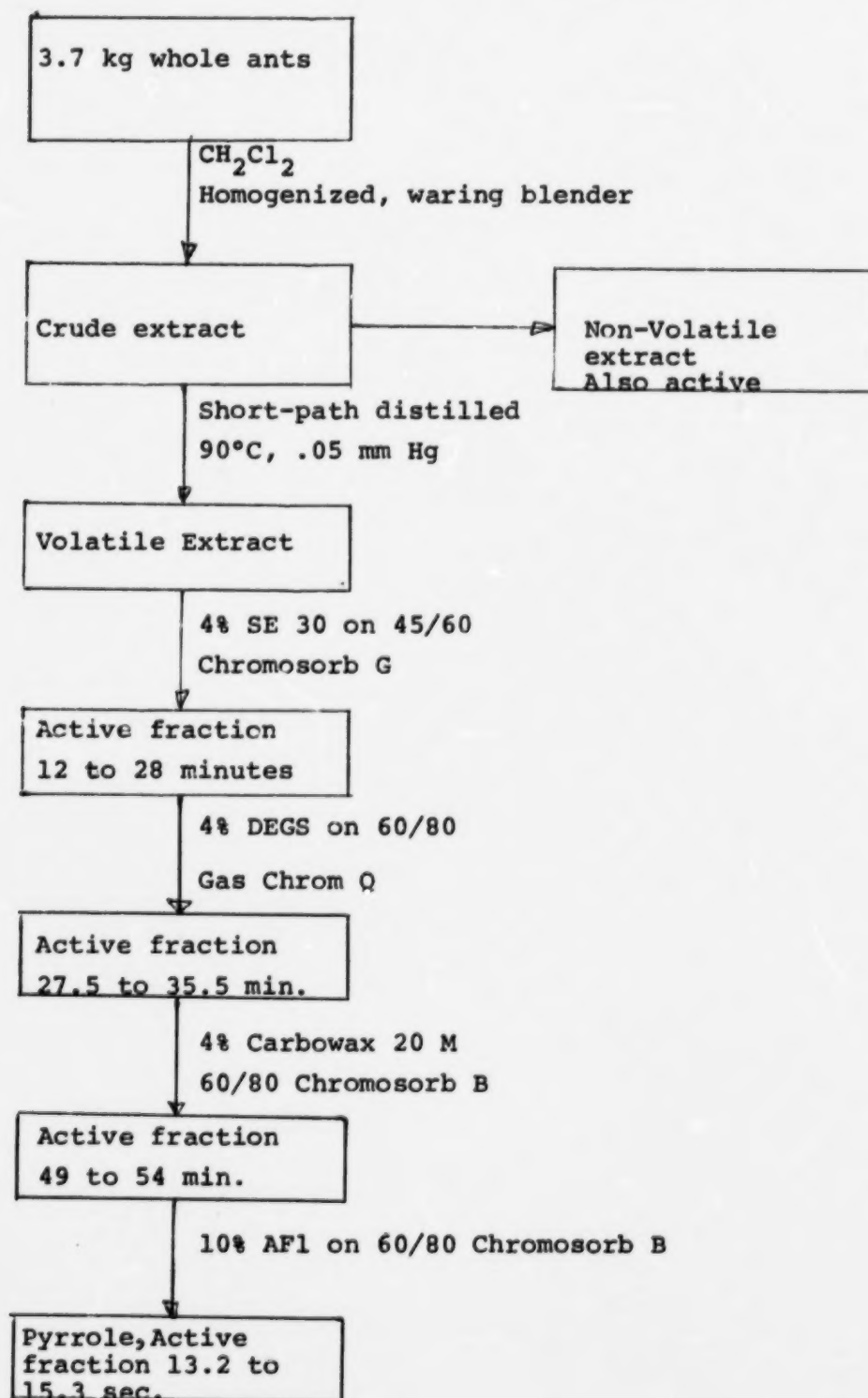
A laboratory bioassay was developed to further confirm the presence of trail pheromone activity. 100 μ l volumes of crude extract from the poison sacs of the ants in carbon tetrachloride were placed on a paper describing a circle six inches in diameter. Sets of minor workers, ten per set, were allowed to crawl about on the paper. The number that travelled at least six inches around the inscribed circle were said to give a positive response.

2. Isolation of the Volatile Trail Pheromone.

3.7 Kilograms of worker ants of all sizes obtained from Grand Parish Louisiana were homogenized in a Waring blender using methylene chloride as a solvent. The crude extract was short-path distilled at 90°C at 0.05 mm Hg. The volatile extract was collected and subjected to gas liquid chromatography. All gas chromatographic separations were monitored by the bioassay procedure described above. The volatile extract was first chromatographed on 4% SE-30 on

Figure 2

Isolation of Volatile Trail Pheromone of Atta texana

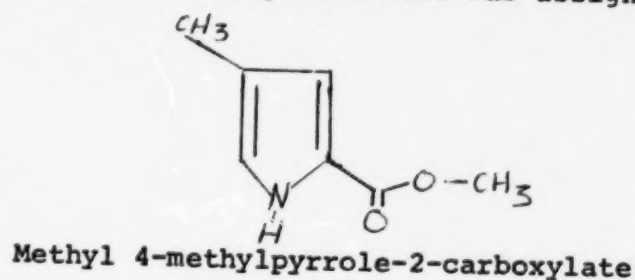


chromosorb G 45/60 mesh, 1 meter x 8 mm, He flow rate 50 cm³/min. Fraction 2 collected from 12 to 28 minutes elicited strong trail pheromone activity. Fractions 1,3 and the backflush showed only traces of activity. Fraction 2 was rechromatographed on 4% diethylene glycol succinate on Gas Chrom Q 60/80 mesh, 2 meters x 4 mm, He flow rate 20 cm³/min, 142° isothermal. The fraction collected between 27.5 and 35.5 min. was found to be highly active. This fraction was rechromatographed on 4% Carbowax 20M on Chromosorb G 60/80 mesh, 7.3 meters x 2.4 mm, He flow rate 25 cm³/min, 170°C isothermal. A major component between 49 and 54 min. gave strong trail pheromone activity. This active component was rechromatographed on 10% QF-1 on Chromosorb G 60/80 mesh, 3 m x 2.4 mm, He flow rate 20 cm³/min, 130° isothermal. A single peak collected between 13.2 and 15.3 min. showed strong trail pheromone activity. A total of 150 µg of the pure compound was collected.

3. Identification of the Volatile Trail Pheromone.

The high resolution mass spectrum showed the active compound to have a molecular weight of 139.0640 corresponding to a molecular formula; C₇H₉O₂N. The IR spectrum showed N-H stretching bands at 3465 cm⁻¹ and 3320 cm⁻¹ and carbonyl absorption at 1695 cm⁻¹. The NMR spectrum showed a three proton singlet at 7.86 τ and one at 6.16 τ corresponding to a methyl group attached to a pyrrole ring and the methyl group of a methyl ester. One proton overlapping multiplets at 3.32 τ and 3.38 τ correspond to the ring protons and a broad signal

from 1.2 τ to 0.4 τ was assigned the proton on the nitrogen atom. From these data, the following structure was assigned:



4. Synthesis and Biological Activity of Trail Pheromone.

Methyl 4-methylpyrrole-2-carboxylate was synthesized by esterification of 4-methylpyrrole-2-carboxylic acid using diazomethane. All spectral and G.L.C. analyses showed the synthetic material to be identical to the naturally occurring material.

Laboratory bioassays with the synthetic material showed the threshold level for minor workers to be 0.08×10^{-12} grams/cm. (3.48×10^8 molecules/cm.).

A practical synthesis of the pyrrole was devised by P. E. Sonnet, U.S.D.A., Beltsville, Md. (Sonnet, 1972). Sonnet in collaboration with J. C. Moser prepared and tested a number of analogues of methyl-4-methylpyrrole-2-carboxylate (Sonnet and Moser, 1972); a number of analogues were active, and some interesting correlations of structure with activity were found. M. S. Blum, University of Georgia, in collaboration with J. C. Moser is investigating the extent of cross attraction of other ant species to methyl 4-methylpyrrole-2-carboxylate (Blum and Moser, 1972). The pyrrole has been supplied to a number of investigators, and reports of further studies should be forthcoming.

C. Isolation Work on the Non-Volatile Trail Pheromone of *Atta texana*.

1. Bioassay and Evidence for the Presence of a Non-Volatile Trail Pheromone.

The bioassay for the non-volatile trail pheromone uses the same bioassay technique that is employed for the volatile-trail pheromone.

The evidence for a non-volatile trail pheromone is based on the following observations: Workers circled on artificial trails that had been kept in darkness at room temperature for six days, but only partially circled trails kept for 12 days. In contrast, trails were circled that had been held under a vacuum of 0.4 mm of mercury for 4 months. It appears that the non-volatile fraction gradually decomposed, probably by oxidation (Moser and Silverstein, 1967).

2. Attempted Isolation of a Non-Volatile Trail Pheromone.

A chromatography column composed of 600 grams of Gallard-Schlesinger Silica gel, (90-200 mesh) was placed in a water jacketed glass column, 2 1/8" O.D. The bed length was 50 cm. On top of the silica gel was placed 2 inches of sea sand. The silica gel was packed wet using benzene that had been dried over potassium carbonate.

To the column was added 45 grams (equivalent to 1723 whole ants) of non-volatile ant extract. The following four fractions were collected:

<u>Fraction</u>	<u>Eluent</u>	<u>Color</u>	<u>Weight</u>
WN1	950 ml. rgt. benzene	green	26.36 grams
WN2	450 ml. Et ₂ O	yellow	11.96 grams
WN3	850 ml. rgt. MeOH	red-brown	6.15 grams
WN4	500 ml. rgt. MeOH	brown	0.61 grams
	Column allowed to run dry		

Bioassays of each of the above fractions at 3 different concentration levels (10 fold differences in concentrations) were sent to our collaborating entomologist, Dr. John Moser. The samples were coded to prevent bias. Solvent blanks were placed at random throughout the samples to make sure that ants were not responding to impurities in the solvent. Concentrations are based on whole ant equivalents.

<u>Fraction</u>	<u>Concentration</u>	<u>Volume</u>	<u>Response</u>
X ₁ chloroform		10μl	-
X ₂ WN1	0.08 mg/μl		-
X ₃ WN2	0.08 mg/μl	"	-
X ₄ WN3	0.80 mg/μl	"	very active
X ₅ chloroform			-
X ₆ WN	0.08 mg/μl	"	slightly active
X ₇ WN2	0.80 mg/μl	"	-
X ₈ chloroform		"	-
X ₉ WN1	0.0 mg/μl	"	-
X ₁₀ WN4	0.80 mg/μl	"	-
X ₁₁ chloroform		"	-
X ₁₂ WN	0.80 mg/μl	"	slightly active

<u>Fraction</u>	<u>Concentration</u>	<u>Volume</u>	<u>Response</u>
X ₁₃ WN2	8.0 mg/μl	"	-
X ₁₄ WN1	0.80 mg/μl	"	-
X ₁₅ chloroform		"	-
X ₁₆ WN	8.0 mg/μl	"	active
X ₁₇ WN3	8.0 mg/μl	"	-
X ₁₈ WN4	8.0 mg/μl	"	-
X ₁₉ WN4	0.08 mg/μl	"	-
X ₂₀ WN3	0.08 mg/μl	"	active

WN corresponds to the crude non-volatile extract prior to any chromatography. It signifies that non-volatile activity and was used to monitor the activity from bioassay to bioassay which varied depending on the disposition of the ants.

Fraction WN3 which was the only active fraction was rechromatographed on 100 grams of silica gel, (90-200 mesh). Its bed length was 42 cm after it was packed wet using ether. The following 3 fractions were collected:

<u>Fraction</u>	<u>Weight</u>	<u>Eluent</u>	<u>Color</u>
WN3A	4.49 grams	300 ml Et ₂ O	Orange-red semi-crystalline
WN3B	0.36 grams	150 ml MeOH	Yellow crystalline
WN3C	0.79 grams	150 ml MeOH	Yellow-orange oil

Bioassays on the above fractions gave the following results:

<u>Fraction</u>	<u>Concentration</u>	<u>Volume</u>	<u>Response</u>
X ₁ chloroform		10μl	-
X ₂ WN3C	0.08 mg/μl	"	-
X ₃ WN3A	0.08 mg/μl	"	very active
X ₄ WN3B	0.08 mg/μl	"	-
X ₅ chloroform		"	-
X ₆ WN3C	0.08 mg/μl	"	-
X ₇ WN3A	0.80 mg/μl	"	very active
X ₈ WN3	0.08 mg/μl	"	slightly active
X ₉ chloroform		"	-
X ₁₀ chloroform		"	-
X ₁₁ WN3B	0.80 mg/μl	"	-
X ₁₂ WN3	0.80 mg/μl	"	very active

From the bioassay results, WN3A appeared to contain all of the activity.

The active fraction was rechromatographed on 100 grams of 90-200 mesh silica gel and the following fractions collected:

<u>Fraction</u>	<u>Weight</u>	<u>Volume and type of Eluent</u>
WN3A1	1.19 grams	150 ml of benzene
WN3A2	2.28 grams	50 ml of benzene
		100 ml 1% ether in benzene
		100 ml 2% ether in benzene
		100 ml 5% ether in benzene
		" " " " " "
		" " " " " "
		" " " " " "

<u>Fraction</u>	<u>Weight</u>	<u>Volume and type of Eluent</u>
WN3A3	1.17 grams	100 ml 10% ether in benzene " " " " " "
		100 ml 20% ether in benzene " " " " " "
WN3A4	0.81 grams	100 ml 50% ether in benzene " " " " " "
		100 ml ether " " " " " "
WN3A5	nil	100 ml MeOH 100 ml MeOH to column dryness

Bioassays on the above five fractions showed WN3A3 to be most active with 75% of the ants giving a positive trail following response. The active fraction was spotted and developed for a distance of 15 cm on a pre-coated silica gel plate (Brinkmann 0.25 mm silica gel HF 254 coated on aluminum) containing both high and low U.V. fluorescent indicators. The developing solvent was ether. The developed plate was observed under high and low wavelength U.V. light and then sprayed with vanillin-sulfuric acid indicator to develop any spots not observed under U.V. light. The developed chromatogram showed a major spot at an R_f of 0.65 which showed up under short wave U.V. and was red when sprayed. A smaller spot at R_f 0.76 was observed under high U.V. light. Trace components (pink when sprayed) at R_f 0.38 and at the origin were also present.

WN3A3 was rechromatographed on 100 grams of Brinkmann silica gel, 90-200 mesh packed wet using benzene. The following fractions were taken:

<u>Fraction</u>	<u>Weight</u>	
WN3A3A	0.22 grams	500 ml 5% Ether in benzene 100 ml 10% Ether in benzene
WN3A3B	0.30 grams	100 ml 10% Ether in benzene 50 ml 15% Ether in benzene
WN3A3C	0.40 grams	50 ml 15% Ether in benzene
WN3A3D	0.16 grams	250 ml pure ether
WN3A3E	0.10 grams	150 ml methanol

Bioassay results showed WN3A3A and WN3A3B to be active with WN3A3A giving a 100% activity response to 0.8 mg/ μ l ant equivalence.

WN3A3A and WN3A3B were spotted on T.L.C. and developed using same conditions as described above. The T.L.C. showed the fractions to possess the same two spots previously described, R_f 0.65 and 0.76. The fractions were recombined into 25 ml of absolute methanol and placed in the refrigerator overnight. The white crystalline material that deposited overnight was collected and recrystallized 3 times from hot methanol. The crystals were dried in an oven at 70°C for 30 minutes and their melting point taken (M.P. 110 to 114°C). T.L.C. of the material showed it to correspond to the red spot at R_f 0.65. An IR of the material showed it to have a spectrum very similar to β sitosterol. Bioassays on the material at several different concentrations showed the material to be inactive, however, bioassay of the mother liquor

showed it to still be highly active. T.L.C. showed the fraction to be composed of a U.V. spot at R_f 0.72, a spot at R_f 0.51 due to residual crystalline material and small amounts of streaked material from R_f 0 to 0.32.

The combined mother liquor from the combination of WN3A3A and WN3A3B (now called WN3A3 (A+B)M) was fractionated on Brinkmann 90-200 mesh silica gel. Fourteen fractions were taken:

<u>Fraction</u>	<u>Eluent</u>	<u>Volume Used</u>
WN3A3 (A+B)M1	3% ether in benzene	100 ml
WN3A3 (A+B)M2	"	"
WN3A3 (A+B)M3	"	50 ml
WN3A3 (A+B)M4	"	"
WN3A3 (A+B)M5	"	100 ml
WN3A3 (A+B)M6	"	"
WN3A3 (A+B)M7	4% ether in benzene	"
WN3A3 (A+B)M8	5% ether in benzene	"
WN3A3 (A+B)M9	7% ether in benzene	"
WN3A3 (A+B)M10	10% ether in benzene	"
WN3A3 (A+B)M11	25% ether in benzene	"
WN3A3 (A+B)M12	50% ether in benzene	"
WN3A3 (A+B)M13	100% ether	"
WN3A3 (A+B)M14	100% MeOH	"

Bioassay of the above fractions showed WN3A3 (A+B)M6 to be weakly active. WN3A3 (A+B)M6 (35 mg) was chromatographed on a high resolution liquid column packed with Bio Sil A (Bio Rad Laboratories) 10 to 30 micron particle size to a height of 50.4 cm. 138 fractions

were collected using benzene-ether (25-75) as a solvent system.

<u>Fraction</u>	<u>Vial Numbers</u>	<u>Weight</u>
WN3A3 (A+B) M6A	1-31	nil
WN3A3 (A+B) M6B	32-60	18.9 mg
WN3A3 (A+B) M6C	61-64	6.7 mg
WN3A3 (A+B) M6D	65-77	2.9 mg
WN3A3 (A+B) M6E	78-82	1.2 mg
WN3A3 (A+B) M6F	83-96	7.8 mg
WN3A3 (A+B) M6G	97-130	2.8 mg
WN3A3 (A+B) M6H	material removed with MeOH	5.1 mg

When the samples were bioassayed, WN3A3(A+B)M6D was active. T.L.C. on silica gel showed the fraction to consist of one U.V. spot. The fraction also showed one spot on Woelm alumina G (neutral) containing 10% gypsum. When a fraction of the active material was chromatographed on T.L.C. using alumina, recollected and bioassayed; no activity was obtained.

It became apparent at this point that our bioassay results were not very reliable. The biological activity of the U.V. spot was erratic with no consistency from bioassay to bioassay.

D. The Volatile Trail Pheromone of *Atta cephalotes*.

1. Collection and Initial Separation.

A total of 2.3 kg. of *Atta cephalotes* from Costa Rica were collected and sent to us over a period of one year. The heads were removed from the abdomens and thorax by freezing the

whole ants at -50°C . They were then shaken while still frozen and the heads broke away from the thorax and abdomen. The ants were placed on a piece of aluminum foil and the heads and abdomens were separated manually.

2. Initial Bioassay Work Location of the Trail Pheromone Activity by Gas-Liquid Chromatography.

15.74 Grams of ants (less heads) were homogenized in four extractions using the Waring blender and 700 ml. of methylene chloride as solvent. The extract was dried over 15 grams of anhydrous potassium carbonate for 24 hours. The dried extract was transferred and the majority of the solvent removed under light vacuum.

The extract was then placed in a short path distillation apparatus; the last traces of solvent removed under aspirator vacuum. The remaining yellow oil was short-path distilled at 50°C and a pressure of 10^{-2} to 10^{-3} mm Hg for two hours. The temperature was then increased to 60°C and the distillation continued for another hour followed by a temperature increase to 70°C for 45 minutes and a final increase to 80°C for an additional 45 minutes.

The volatile material was removed from the cold finger using chloroform and the non-volatile material recollected using chloroform.

Samples of both the volatile and the non-volatile extract were sent to Costa Rica for bioassay. Both the volatile and non-volatile portions of the total ant extract were active.

The entire volatile extract was chromatographed on 5% SE-30 on Chromosorb W 60/80 mesh, 2 1/2 ft. x 1/4 in. The column

was initially at 80°C. Six minutes after injection of the sample, the column was temperature programmed at a rate of 10°C/min to a final temperature of 255°C. The following 5 fractions were taken:

<u>Fraction</u>	<u>Retention Time</u>
WGV-1	0 to 5 min. 24 sec.
WGV-2	5 min. 24 sec. to 14 min. 24 sec.
WGV-3	14 min. 24 sec. to 17 min. 24 sec.
WGV-4	17 min. 24 sec. to 19 min. 48 sec.
WGV-5	19 min. 48 sec. to 33 min.

Bioassay of the above fractions showed WGV-2 to be highly active. It was the only active fraction.

3. Large Scale Work-up of Atta cephalotes Gaster Material.

1.1 Kilograms of ant material composed of abdomens, legs and the thorax were homogenized in the Waring blender using fractionally distilled CHCl_2 as the solvent. The extract was dried over anhydrous sodium sulfate and the solvent removed slowly by fractional distillation at atmospheric pressure.

The crude concentrated extract was divided into two portions and both portions were short-path distilled at 60°C and 0.15 mm Hg. for 2 1/2 hours. The volatile material was removed and collected in 5 ml of methylene chloride.

The entire volatile extract was chromatographed on 5% SE-30 on Chromosorb W 60/80 mesh, 2 1/2 ft. x 1/4 in. fitted

with precolumn and backflush system. The same column conditions as previously described were used for this chromatographic separation. The following fractions were collected:

<u>Fraction</u>	<u>Collection Time</u>
WGV-1	0 to 5 min. 36 sec.
WGV-2	5 min. 36 sec. to 16 min.
WGV-3	16 min. to 23 min. 12 sec.
WGV-4	23 min. 12 sec. to 48 min.
Backflush	48 min. to 96 min.

Fraction WGV-2, shown to be active from preliminary bioassay studies is presently being chromatographed on a 4% Carbowax 20M column.

References

1. Blum, Murray S., Padovani, Flavio, and Amante, Elpidio, "Alkanones and Terpenes in the Mandibular Glands of Atta Species (Hymenoptera: Formicidae)," Comp. Biochem. Physiol., 26, pp 291-299 (1968).
2. Blum, Murray S., Warter, Stuart L., Traynham, James G., "Chemical Releasers of Social Behaviour-VI. The Relation of Structure to Activity of Ketones as Releasers of Alarm for Iridomyrmex Pruinosus (Roger)," J. Insect Physiol., 12, pp 419-427 (1966).
3. Blum, Murray, S., Moser, John C., and Cordero, A. D., "Chemical Releasers of Social Behavior. II. Source and Specificity of the Odor Trail Substances in four Attine Genera (Hymenoptera: Formicidae)," Psyche, 71, pp 1-7 (1964).
4. Blum, M. S., and Portocarrero, C. A., "Chemical Releasers of Social Behavior. X. An Attine Trail Substance in the Venom of a Non-Trail Laying Myrmicine, Daceton Armigerum (Labreille)," Psyche, 73, pp 150-155 (1966).
5. Blum, M. S., and Moser, J. C., "Cross Attraction Studies with the Trail Pheromone of Atta texana. In Preparation.

6. Crewe, Robin M., Blum, Murray S., "Alarm Pheromones of the Attini: Their Phylogenetic Significance," J. Insect Physiol., 18, pp 31-42 (1972).
7. McGurk, D. J., Frost, Jennifer, Eisenbraun, E. J., Vick, K., Drew, W. A., and Young, J., "Volatile Compounds in Ants: Identification of 4-methyl-3-heptanone from Pogonomyrmex Ants," J. Insect Physiol., 12, pp 1435-1441 (1966).
8. Moser, J. C., Brownlee, R. G., and Silverstein, R. M., "The Alarm Pheromones of the Ant Atta texana," J. Insect Physiol., 14, pp 529-535 (1968).
9. Moser, J. C., "Trail Marking of the Texas Leaf-Cutting Ant: Source and Potency," Science, 140, p 1228 (1963).
10. Moser, J. C., Silverstein, R. M., "Volatility of the Trail Marking Substance of the Town Ant," Nature, 215, pp 206-207 (1967).
11. Sonnet, P. E., and Moser, J. C., "Analogues of the Trail Pheromone of Atta Texana." In preparation.
12. Sonnet, P. E., "Synthesis of the Trail Marker of the Texas Leaf-Cutting Ant, Atta texana (Buckley)," J. Med. Chem., 15, pp 97-98 (1972).

13. Tumlinson, J. H., Silverstein, R. M., Moser, J. C., Brownlee, R. G., and Ruth, J. M., "Identification of the Trail Pheromone of the Leaf-Cutting Ant, Atta texana," Nature, 234, pp 348-349 (1971).
14. Tumlinson, J. H., Moser, J. C., Silverstein, R. M., Brownlee, R. G., and Ruth, J. M., "A Volatile Trail Pheromone of the Leaf-Cutting Ant, Atta texana," J. Insect Physiol., 18, pp 809-814 (1972).
15. Weber, Neal A., "Fungus-Growing Ants," Science, 153, pp 587-604 (1966).